Dkt. #792-A-PCT-US

# *Application for United States Letters Patent*

**To all whom it may concern:**

Be it known that WEI, Guangwen, GUO, Rongbing and ZHANG, Renhuai

have invented certain new and useful improvements in

**RECOMBINANT SUPER-COMPOUND INTERFERON**

of which the following is a full, clear and exact

description

# RECOMBINANT SUPER-COMPOUND INTERFERON

The application is a continuation-in-part application of International Patent Application No. PCT/CN02/00128, filed on 28 February 2002, which claims priority of Chinese Application No. 01104367.9, filed on 28 February 2001, the contents of which are incorporated by reference here into this application.

Throughout this application, various references are referred to. Disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

## FIELD OF THE INVENTION

This invention is related to a recombinant super-compound interferon (rSIFN-co) with changed spatial configuration. One characteristic of rSIFN-co in this invention is that it cannot only inhibit DNA (deoxyribonucleic acid) duplication of the hepatitis B virus but also the secretion of HBsAg and HBeAg.

## BACKGROUND OF THE INVENTION

rSIFN-co is a new interferon molecule constructed with the most popular conservative amino acid found in natural human α-IFN subtypes using genetic engineering methods. United States Patent Nos. 4,695,623 and 4,897,471 have described it. rSIFN-co had been proved to have broad-spectrum IFN activity and virus- and tumor-inhibition and natural killer cell activity. United States Patent No. 5,372,808 by Amgen, Inc. addresses treatment rSIFN-co. Chinese Patent No. 97193506.8 by Amgen, Inc. addresses re-treatment of rSIFN-co on hepatitis C. Chinese Patent No. 98114663.5 by Shenzhen Jiusheng Bio-engineering Ltd. addresses treatment of rSIFN-co on hepatitis B and hepatitis C.

The United States Food and Drug Administration (FDA)

authorized Amgen to produce rSIFN-co with *E. Coli.* for clinical hepatitis C treatment at the end of 1997.

Hepatitis B patients can be identified when detecting HBsAg and the HBeAg. α-IFN is commonly used in clinics to treat hepatitis B. IFN binds superficial cell membrane receptors, inhibiting DNA and RNA (ribonucleic acid) duplication, including inducing some enzymes to prevent duplication of the virus in hepatitis-infected cells. All IFNs can inhibit only the DNA duplication of viruses, not the e and s antigen.

This disclosure describes recombinant super-compound interferon, method to produce the same and uses thereof.

**SUMMARY OF THE INVENTION**

This invention provides a recombinant super-compound interferon or an equivalent thereof with changed spatial configuration. An equivalent is a molecule which is similar in function to the super-compound interferon. The super-compound interferon possesses anti-viral or anti-tumor activity. This invention also provides an artificial gene codes for the super-compound interferon or its equivalent.

This invention provides a process for production of recombinant super-compound interferon comprising introducing an artificial gene with selected codon preference into an appropriate host, culturing said introduced host in an appropriate condition permitting expression of said super-compound interferon and harvesting the expressed super-compound interferon.

This invention provides a composition comprising the recombinant super-compound interferon or its equivalent and a suitable carrier. This invention further provides a pharmaceutical composition comprising the recombinant super-compound interferon or its equivalent and a pharmaceutically acceptable carrier.

This invention provides a method for treating viral diseases or tumor in a subject comprising administering to the subject an effective amount of the super-compound interferon or its equivalent.

This invention provides the above-described method wherein super-compound interferon was administered via oral, vein injection, muscle injection, peritoneal injection, subcutaneous injection, nasal, mucosal administration, by inhalation via an inspirator.

## DETAILED DESCRIPTION OF THE FIGURES

**Figure 1.** rSIFN-co cDNA sequence designed according to *E. Coli.* codon usage and deduced rSIFN-co amino acid sequence

**Figure 2.** Sequence of another super-compound interferon

**Figure 3.** Diagram of pLac T7 cloning vector plasmid

**Figure 4.** Diagram of pHY-4 expression vector plasmid

**Figure 5.** Construction process of expression plasmid pHY-5

**Figure 6-A.** Circular Dichroism spectrum of Infergen®
Spectrum range: 250nm - 190nm
Sensitivity: 2 m°/cm
Light path: 0.20 cm
Equipment: Circular Dichroism J-500C
Samples: contains 30$\mu$g/ml IFN-con1, 5.9 mg/ml of NaCl and 3.8 mg/ml of $Na_2PO_4$, pH7.0.

Infergen® (interferon alfacon-1), made by Amgen Inc., also known as consensus interferon, is marketed for the treatment of adults with chronic hepatitis C virus (HCV) infections. It is currently the only FDA approved, bio-optimized interferon developed through rational drug design and the only interferon with data in the label specifically for non-responding or refractory patients. InterMune's sales force re-launched Infergen® in January 2002 with an active campaign to educate U.S. hepatologists about the safe and appropriate use of Infergen®, which represents new hope for the more than 50 percent of HCV patients who fail other currently available therapies. See http://www.intermune.com/wt/itmn/infergen, 8/27/2003

**Figure 6-B.** Circular Dichroism spectrum of Infergen® From Reference [Journal of Interferon and Cytokine Research. 16:489-499(1996)]

**Figure 6-C.** Circular Dichroism spectrum of rSIFN-co
Spectrum range: 320nm-250nm
Sensitivity: 2 m°/cm
Light path: 2cm
Equipment: Circular Dichroism J-500C
Samples: contains 0.5mg/ml rSIFN-co, 5.9 mg/ml of NaCl and 3.8 mg/ml of $Na_2PO_4$, pH7.0.

**Figure 6-D.** Circular Dichroism spectrum of rSIFN-co
Spectrum range: 250nm - 190nm
Sensitivity: 2 m°/cm
Light path: 0.20 cm
Equipment: Circular Dichroism J-500C
Samples: contains 30$\mu$g/ml rSIFN-co, 5.9 mg/ml of NaCl and 3.8 mg/ml of $Na_2PO_4$, pH7.0.

Clearly, as evidenced by the above spectra, the secondary or even tertiary structure of rSIFN-co is different from Infergen®.

**DETAILED DESCRIPTION OF THE INVENTION**

This invention provides a recombinant super-compound interferon or an equivalent thereof with changed spatial configuration. This invention reveals that protein with same primary sequence might have different biological activities. As illustrated in the following example, this invention disclosed two proteins with identical amino acid sequence but with different activities. This activity may sometimes become improved efficacy and sometimes, the protein with changed spatial configuration would reveal new function.

An equivalent is a molecule which is similar in function to the compound interferon. An equivalent could be a deletion, substitution, or replacement mutant of the original sequence. Alternatively, it is also the intention of this invention to cover mimics of the recombinant super-compound interferon. Mimics could be a peptide, polypeptide or a small chemical entity.

The interferon described herein includes but is not limited to interferon $\alpha$, $\beta$, or $\omega$. In an embodiment, it is IFN-1a, IFN-2b or other mutants.

In an embodiment, the super-compound interferon disclosed has higher efficacy than the interferon described in U.S. Patent Nos. 4,695,623 or 4,897,471. This super-compound interferon is believed to have unique secondary or tertiary structure. (See e.g. Figure 6)

The super-compound interferon described herein has spatial structure change(s) resulting from the changes of its production process.

The above-described super-compound interferon may be produced by a high efficiency expression system which uses

a special promoter. In an embodiment, the promoter is $P_{BAD}$. As it could be easily appreciated by other ordinary skilled artisan, other inducible promoter, such as heat shock promoter, may be used in this invention.

The super-compound interferon may also be produced with its gene as artificially synthesized cDNA with adjustment of its sequence from the wild-type according to codon preference of *E. Coli*. Extensive discussion of said codon usage (preference) may be found in U.S. Patent No. 4,695,623. See e.g. column 6, line 41 - column 7, line 35

The above described super-compound interferon possesses anti-viral or anti-tumor activity and therefore useful in preventing and treating viral diseases, tumors or cancers.

The virus diseases include but are not limited to hepatitis A, hepatitis B, hepatitis C, other types of hepatitis, infections caused by Epstein-Barr virus, Cytomegalovirus, herpes simplex viruses, other herpes viruses, papovaviruses, poxviruses, picornaviruses, adenoviruses, rihnoviruses, human T cell leukaemia viruses I, human T cell leukaemia viruses II, or human T cell leukemia viruses III.

Therefore, this invention provides a method for inhibiting virus replication or virus infected cells by contacting said virus or infected cells with an effective amount of the super-compound interferon or its equivalent. This super-compound interferon is useful in preventing or treating the following cancers or tumors:

| | | |
|---|---|---|
| Cancer | Skin Cancer | Basal Cell Carcinoma |
| | | Malignant Melanoma |
| | Renal cell carcinoma | |
| | Liver Cancer | |

| | | |
|---|---|---|
| | Thyroid Cancer | |
| | Rhinopharyngeal Cancer | |
| | Solid Carcinoma | Prostate Cancer |
| | | Tummy Cancer |
| | | Esophagus Cancer |
| | | Recta Cancer |
| | | Pancreas Cancer |
| | | Mammary Cancer |
| | Ovarian Cancer & Superficial Bladder Cancer | |
| | Hemangioma | |
| | Epidermoid Carcinoma | Cervical Cancer |
| | | Non-small Cell Lung Cancer |
| | | Small Cell Lung Cancer |
| | | Glioma |
| Malignant Hemal Disease | Leucocythemia | Acute Leucocythemia |
| | | Chronic Leucocythemia |
| | Chronic Myelocytic Leukemia | |
| | Hairy Cell Leukemia | |
| | Lymphadenoma | |
| | Multiple Myeloma | |
| | Polycythemia Vera | |
| Others | Kaposi's Sarcoma | |

Accordingly, this invention provides a method for inhibiting tumor or cancer cell growth by contacting the super-compound interferon or its equivalent with said tumor or cancer cells. In a further embodiment, the super-

compound interferon inhibits the DNA duplication and secretion of HBsAg and HBeAg of Hepatitis B Virus.

This invention also provides an artificial gene codes for the super-compound interferon or its equivalent. It is within the ordinary skill to design an artificial gene. Many methods for generating nucleotide sequence and other molecular biology techniques have been described previously. See for example, Joseph Sambrook and David W. Russell, Molecular Cloning: A laboratory Manual, December 2000, published by Cold Spring Harbor Laboratory Press.

This invention provides a vector comprising the gene which codes for the super-compound interferon or its equivalent.

This invention provides an expression system comprising the vector comprising the gene which codes for the super-compound interferon or its equivalent. The cells include but are not limited to prokaryotic or eukaryotic cells.

This invention also provides a host cell comprising the vector comprising the gene which codes for the super-compound interferon or its equivalent.

This invention provides a process for production of recombinant super- compound interferon comprising introducing an artificial gene with selected codon preference into an appropriate host, culturing said introduced host in an appropriate condition for the expression of said compound interferon and harvesting the expressed compound interferon.

The process may comprise extraction of super-compound interferon from fermentation broth, collection of inclusion body, denaturation and renaturation of the harvested protein.

The process may maintain the high efficacy even when the super-compound interferon is used with an agent and in a particular concentration. The process also comprises separation and purification of the super-compound interferon. The process further comprises lyophilization of the purified super-compound interferon. The process comprises production of liquid injection of super-compound interferon.

This invention also provides the produced super-compound interferon by the above processes.

This invention provides a composition comprising the recombinant super-compound interferon or its equivalent and a suitable carrier.

This invention provides a pharmaceutical composition comprising the recombinant super-compound interferon or its equivalent and a pharmaceutically acceptable carrier.

This invention provides a method for treating viral diseases or tumor in a subject comprising administering to the subject an effective amount of the super-compound interferon or its equivalent.

This invention provides the above-described method wherein the viral diseases is hepatitis A, hepatitis B, hepatitis C, other types of hepatitis, infections of viruses caused by Epstein-Barr virus, Cytomegalovirus, herpes simplex viruses, or other type of herpes viruses, papovaviruses, poxviruses, picornaviruses, adenoviruses, rihnoviruses, human T cell leukaemia viruses I, or human T cell leukaemia viruses II, or human T cell leukemia virus III.

This invention provides the above-described method wherein super-compound interferon was administered via oral, vein

injection, muscle injection, peritoneal injection, subcutaneous injection, nasal, mucosal administration, by inhalation via an inspirator.

This invention provides the above-described method wherein super-compound interferon was administered following the protocol of injection 9 μg or 15 μg per day, 3 times a week, total 24 weeks.

It was surprising to find that rSIFN-co, the spatial structure of which has been changed, is not only a preparation to inhibit the DNA duplication of hepatitis B, but to inhibit the secretion of HBsAg and HBeAg on 2.2.15 cells.

One objective of this invention is to offer a preparation of rSIFN-co to directly inhibit the DNA duplication of hepatitis B viruses and the secretion of HBeAg and HBsAg of hepatitis B and decrease them to normal levels.

In one of the results of this invention, rSIFN-co was produced with recombinant techniques. On the condition of fixed amino acid sequence, the IFN DNA was redesigned according to the *E. Coli.* codon usage and then the rSIFN-co gene was artificially synthesized. rSIFN-co cDNA was cloned into the high-expression vector of *E. Coli.* by DNA recombinant techniques, and a high expression of rSIFN-co was gained by using of induce/activate-mechanism of L-arabinose to activate the transcription of $P_{BAD}$ promoter.

Compared with usual thermo-induction, pH induction and IPTG induction systems of genetic engineering, arabinose induction/activation system has some advantages: (1) Common systems relieve promoter function by creating a "derepression" pattern. Promoters then induce downstream gene expression. So temperature and pH change and the addition of IPTG cannot activate promoters directly. In the system disclosed herein, L-arabinose not only deactivates

and represses but also activates the transcription of $P_{BAD}$ promoter which induce a high expression of rSIFN-co. Therefore, the arabinose induction/activation system is a more effective expression system. (2) The relation between Exogenous and L-arabinose dosage is linearity. This means the concentration of arabinose can be changed to adjust the expression level of the exogenous gene. Therefore, it is easier to control the exogenous gene expression level in *E. Coli.* by arabinose than by changing temperature and pH value. This characteristic is significant for the formation of inclusion bodies. (3) L- arabinose is resourceful cheap and safe, which, on the contrary, are the disadvantages of other inducers such as IPTG.

This embodiment creates an effective and resistant rSIFN-co-expressing *E. Coli.* engineering strain with an L-arabinose induction/activation system. The strain is cultivated and fermented under suitable conditions to harvest the bacterial bodies. Inclusion bodies are then purified after destroying bacteria and washing repeatedly. The end result, mass of high-purity, spatial-configuration-changed rSIFN-co protein for this invention and for clinical treatment, was gained from denaturation and renaturation of inclusion bodies and a series of purification steps.

The following are some rSIFN-co preparations: tablets, capsules, oral liquids, pastes, injections, sprays, suppositories, and solutions. Injections are recommended. It is common to subcutaneously inject or vein-inject the medicine. The medicine carrier could be any acceptance medicine carrier, including carbohydrate, cellulosum, adhesive, collapse, emollient, filling, add-dissolve agent, amortization, preservative, add-thick agent, matching, etc.

This invention also provides a pharmaceutical composition comprising the above composition and a pharmaceutically

acceptable carrier.

For the purposes of this invention, "pharmaceutically acceptable carriers" means any of the standard pharmaceutical carriers. Examples of suitable carriers are well known in the art and may include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution and various wetting agents. Other carriers may include additives used in tablets, granules and capsules, etc. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gum, glycols or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well-known conventional methods.

This invention will be better understood from the examples which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

## EXPERIMENTAL DETAILS

### EXAMPLE 1

rSIFN-co is a new interferon molecule constructed according to conservative amino acid in human IFN-α subtype with genetic engineering method. It has been proven that rSIFN-co has broad-spectrum IFN activity, such as high antivirus and tumor inhibition activity, especially for effectively treating hepatitis C.

E. Coli. codon was used to redesign rSIFN-co cDNA and then artificially synthesize cDNA of rSIFN-co from published rSIFN-co DNA sequences and deduced amino acid sequences

(Figure 1).

In order to get pure rSIFN-co protein, rSIFN-co cDNA was cloned into E. Coli. high-expression vector, and L-arabinose, which can activate strong $P_{BAD}$ promoter in vectors, was used to induce high expression of rSlFN-co gene.

**Synthesis of *E. Coli.* cDNA Sequence**

**Redesign of rSIFN-co cDNA sequence**

rSIFN-co cDNA was redesigned according to the codon usage of *E. Coli.* to achieve high expression in *E. Coli.* Deduced amino acid sequence from the redesigned cDNA sequence of rSIFN-co is completely coincidental with primitive amino acid sequence of published rSIFN-co (Figure 1).

**rSIFN-co cDNA sequence synthesis**

**rSIFN-co cDNA 5'-terminus and 3'- terminus semi-molecular synthesis**

Two semi-moleculars can be directly synthesized: rSIFN-co cDNA 5'- terminus 280bp (fragment I) and 3'- terminus 268bp(fragment II) by PCR. There are 41bp overlapping among fragment II and fragment I.

(1) Chemical synthesis oligodeoxynucleotide fragment:

Oligomer A:

5'ATGTGCGACCTGCCGCAGACCCACTCCCTGGGTAACCGTCGTGCTCTGATCCTGCTGGCTCA
GATGCGTCGTATCTCCCCGTTCTCCTGCCTGAAAGACCGTCACGAC3'

Oligomer B:

5'CTGAAAGACCGTCACGACTTCGGTTTCCCGCAGGAGAGGTTCGACGGTAACCAGTTCCAGA
AGCTCAGGCTATCTCCGTTCTGCACGAAATGATCCAGCAGACCTTC3'

Oligomer C:

5'GCTGCTGGTACAGTTCGGTGTAGAATTTTTCCAGCAGGGATTCGTCCCAAGCAGCGGAGGAG
TCTTTGGTGGAGAACAGGTTGAAGGTCTGCTGGATCATTTC3'

Oligomer D:

5'ATCCCTGCTGGAAAAATTCTACACCGAACTGTACCAGCAGCTGAACGACCTGGAAGCTTGCG

TTATCCAGGAAGTTGGTGTTGAAGAAACCCCGCTGATGAAC3'

Oligomer E:

5'GAAGAAACCCCGCTGATGAACGTTGACTCCATCCTGGCTGTTAAAAAATACTTCCAGCGTATCACCCTGTACCTGACCGAAAAAAAATACTCCCCGTGCGCTTGGG3'

Oligomer F:

5'TTATTCTTTACGACGCAGACGTTCCTGCAGGTTGGTGGACAGGGAGAAGGAACGCATGATTTCAGCACGAACAACTTCCCAAGCGCACGGGGAGTATTTTTTTCGGTCAGG3'

PCR I for Fragment I: oligodeoxynucleotide B as template, oligodeoxynucleotide A and C as primers, synthesized 280 bp Fragment I.

PCR I mixture (units: μl)

| | |
|---|---|
| sterilized distilled water | 39 |
| 10×Pfu buffer (Stratagen American Ltd.) | 5 |
| dNTP mixture (dNTP concentration 2.5 mmol/L) | 2 |
| Oligomer A primer (25 μmol/L) | 1 |
| Oligomer C primer (25 μmol/L) | 1 |
| Oligomer B template (1 μmol/L) | 1 |
| Pfu DNA polymerase (Stratagen American Ltd.) (25 U/μl) | 1 |
| Total volume | 50μl |

PCR cycle: 95 I

2m→(95℃45s→65℃1m→72℃1m)×25 cycle→72℃10m→4℃

PCR II for Fragment II: oligodeoxynucleotide E as template, oligodeoxynucleotide D and F as primers, synthesized 268bp Fragment II.

PCR II mixture (units: μl)

| | |
|---|---|
| sterilized distilled water | 39 |
| 10×Pfu buffer (Stratagen American Ltd.) | 5 |
| dNTP mixture (dNTP concentration 2.5mmol/L) | 2 |
| Oligomer D primer (25 μmol/L) | 1 |
| Oligomer F primer (25 μmol/L) | 1 |
| Oligomer E template (1 μmol/L) | 1 |

Pfu DNA polymerase (Stratagen American Ltd.) (25U/μl) 1

Total volume 50μl

PCR cycle: the same as PCR I

**Assembling of rSIFN-co cDNA**

Fragment I and II were assembled together to get the complete cDNA molecular sequence of rSIFN-co using the overlapping and extending PCR method. Restriction enzyme Nde I and Pst I were introduced to clone rSIFN-co cDNA sequence into plasmid.

(1) Chemical synthesis primers

Oligomer G: 5'ATCGGCCATATGTGCGACCTGCCGCAGACCC3'

Oligomer H: 5'ACTGCCAGGCTGCAGTTATTCTTTACGACGCAGACGTTCC3'

(2) Overlapping and extending PCR

| PCR mixture | (units: μl) |
|---|---|
| sterilized distilled water | 38 |
| 10×Pfu buffer (Stratagen American Ltd.) | 5 |
| dNTP mixture (dNTP concentration 2.5mmol/L) | 2 |
| primer G (25 μmol/L) | 1 |
| primer H (25 μmol/L) | 1 |
| *fragment I preduction (1 μmol/L) | 1 |
| *fragment II preduction (1 μmol/L) | 1 |
| Pfu DNA polymerase (Stratagen American Ltd.) (2.5U/μl) | 1 |
| Total volume | 50μ |

*Separate and purify PCR production with StrataPrep PCR purification kit produced by Stratagen American Ltd. And dissolve into sterilized distilled water.

PCR cycle: the same as PCR I

**rSIFN-co gene clone and sequence analysis**

pLac T7 plasmid as cloning vector. pLac T7 plasmid is reconstructed with pBluescript II KS(+) plasmid produced by Stratagen (Figure 3).

Purified PCR production of rSIFN-co cDNA with StrataPrep PCR purification kit. Digest cDNA and pLac T7 plasmid with NdeI and PstI. Run 1% agarose gel electrophoresis and separate these double-digested DNA fragments. Recover 507bp long rSIFN-co DNA fragment and 2.9kb plasmid DNA fragment. Ligate these fragments by T4 DNA ligase to form a recombinant plasmid. Transform $DH_{5\alpha}$competent cells (Gibco) with the recombinant plasmid, culture at 37°C overnight. Identify the positive recombinant colony, named pHY-1.

Run DNA sequencing with SequiTherm™ Cycle Sequencing Kit produced by American Epicentre Technologies Ltd using L1-COR Model 4000L. Primers are T7 and T3 common sequence primer, the DNA sequencing result matches theoretic design.

Purify the rSIFN-co, sequence the N-terminus amino acids, the N-terminus amino acid sequence matches experimental design which is as follows:

N- Cys-Asp-Leu-Pro-Gln-Thr-His-Ser-Leu-Gly-Asn-Arg-Arg-Ala-Leu-

**Construction, transformation, identification, and hereditary stability of expression vector**

**Construction and transformation of expression vector**

Digested *E. Coli.* expression vector pHY-4(see Figure 3) with Nde I to linearize and subsequently digest with Xba I. Run 1% agarose gel electrophoresis, and purify the 4.8kb pHY-4 Nde I -Xba I digest fragment with QIAEX II kit produced by QIAGEN Germany Ltd.

At the same time, the pHY-4 plasmid is double digested with Nde I-Xba I. Run 1% agarose gel electrophoresis and purify the 715bp fragment. Ligate the rSIFN-co and pHY-4 fragments with T4 DNA ligase to construct the recombinant plasmid (See Figure 4). Transform $DH_{5\alpha}$competent cells with the

recombinant plasmid. Spread the transformed cells on LB plate with Amp, 37℃ culture overnight.

**Positive cloning strain screening**

Randomly choose *E. Coli.* colonies from above LB-plate, screening the positive strains containing recombinant vector by endonuclease digesting and PCR analysis. Name one of the positive recombinant plasmid pHY-5, and name the strain containing pHY-5 plasmid PVIII. Amplify and store the positive strain with glycerol in -80℃.

**High expression of rSIFN-co gene in *E. Coli.***

In pHY-5 plasmid, rSIFN-co gene is under control of strong promoter $P_{BAD}$. This promoter is positively and negatively regulated by the product of the gene araC. AraC is a transcriptional regulator that forms a complex with arabinose. In the absence of arabinose, the AraC dimer binds $O_2$ and $I_1$ forming a 210bp loop. This conformation leads to a complete inhibition of transcription. In the presence of arabinose, the dimer is released from $O_2$ and binds $I_1$ and $I_2$ leading to transcription. Arabinose binding deactivates, represses and even activates the transcription of $P_{BAD}$ promoter, which stimulates $P_{BAD}$ inducing high expression of rSIFN-co. rSIFN-co expression level in PVIII is more than 50% of the total *E. Coli.* protein.

**Summary**

RSIFN-CO is a new interferon molecule artificially built according to the conservative amino acid of human α interferons. It has been proven as a effective anti-hepatitis drug. In order to get enough pure rSIFN-co protein, a stable recombinant *E. Coli.* strain which high expresses rSIFN-co protein was constructed.

First, according to published rSIFN-co amino acid sequence,

*E. Coli.* codon was used to synthesize whole cDNA of rSIFN-co. This DNA fragment was sequenced and proved that the 501bp codon sequence and TAA termination codon sequence are valid and identical with theocratic design. Subsequent analysis revealed that the N-terminus amino acid sequence and amino acid composed of rSIFN-co produced by the recombinant strain were both identical to the prediction.

The rSIFN-co cDNA was cloned into *E. Coli.* high-expression vector pHY-4 plasmid to construct the recombinant plasmid pHY-5. *E. Coli.* LMG194 strain was further transformed with pHY-4 plasmid to get stable rSIFN-co high-expression transformant. This transformant was cultured for 30 generations. The heredity of pHY-5 recombinant plasmid in *E. Coli.* LMG194 was normal and stable, and the expression of rSIFN-co was high and steady.

*E. Coli.* LMG194, which contains recombinant pHY-5 plasmid, is actually an ideal high-expression engineering strain.

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**rSIFN-co cDNA SEQUENCE DESIGNED ACCORDING TO E. COLI. CODON USAGE AND DEDUCED rSIFN-co AMINO ACID SEQUENCE**

```
    5'         11         21         31         41         51
+1  M  C  D  L  P  Q  T  H  S  L  G  N  R  R  A  L  I  L  L  A
  1 ATGTGCGACC TGCCGCAGAC CCACTCCCTG GGTAACCGTC GTGCTCTGAT CCTGCTGGCT
    TACACGCTGG ACGGCGTCTG GGTGAGGGAC CCATTGGCAG CACGAGACTA GGACGACCGA

    5'         71         81         91         101        111
+1  Q  M  R  R  I  S  P  F  S  C  L  K  D  R  H  D  F  G  F  P
 61 CAGATGCGTC GTATCTCCCC GTTCTCCTGC CTGAAAGACC GTCACGACTT CGGTTTCCCG
    GTCTACGCAG CATAGAGGGG CAAGAGGACG GACTTTCTGG CAGTGCTGAA GCCAAAGGGC

    5'         131        141        151        161        171
+1  Q  E  E  F  D  G  N  Q  F  Q  K  A  Q  A  I  S  V  L  H  E
121 CAGGAAGAAT TCGACGGTAA CCAGTTCCAG AAAGCTCAGG CTATCTCCGT TCTGCACGAA
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GTCCTTCTTA AGCTGCCATT GGTCAAGGTC TTTCGAGTCC GATAGAGGCA AGACGTGCTT

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    5'          191       201       211       221       231
+1  M  I  Q   Q  T  F  N   L  F  S   T  K  D   S  S  A  A   W  D  E
181 ATGATCCAGC AGACCTTCAA CCTGTTCTCC ACCAAAGACT CCTCCGCTGC TTGGGACGAA
    TACTAGGTCG TCTGGAAGTT GGACAAGAGG TGGTTTCTGA GGAGGCGACG AACCCTGCTT

    5'          251       261       271       281       291
+1  S  L  L   E  K  F  Y   T  E  L   Y  Q  Q   L  N  D  L   E  A  C
241 TCCCTGCTGG AAAAATTCTA CACCGAACTG TACCAGCAGC TGAACGACCT GGAAGCTTGC
    AGGGACGACC TTTTTAAGAT GTGGCTTGAC ATGGTCGTCG ACTTGCTGGA CCTTCGAACG

    5'          311       321       331       341       351
+1  V  I  Q   E  V  G  V   E  E  T  P   L  M  N   V  D  S   I  L  A
301 GTTATCCAGG AAGTTGGTGT TGAAGAAACC CCGCTGATGA ACGTTGACTC CATCCTGGCT
    CAATAGGTCC TTCAACCACA ACTTCTTTGG GGCGACTACT TGCAACTGAG GTAGGACCGA

    5'          371       381       391       401       411
+1  V  K  K  Y  F  Q  R  I  T  L  Y  L  T  E  K  K  Y  S  P  C
361 GTTAAAAAAT ACTTCCAGCG TATCACCCTG TACCTGACCG AAAAAAAATA CTCCCCGTGC
    CAATTTTTTA TGAAGGTCGC ATAGTGGGAC ATGGACTGGC TTTTTTTTAT GAGGGGCACG

    5'          431       441       451       461       471
+1  A  W  E  V  V  R  A  E  I  M  R  S  F  S  L  S  T  N  L  Q
421 GCTTGGGAAG TTGTTCGTGC TGAAATCATG CGTTCCTTCT CCCTGTCCAC CAACCTGCAG
    CGAACCCTTC AACAAGCACG ACTTTAGTAC GCAAGGAAGA GGGACAGGTG GTTGGACGTC

    5'          491       501
+1  E  R  L   R  R  K  E   #
481 GAACGTCTGC GTCGTAAAGA ATAA
    CTTGCAGACG CAGCATTTCT TATT
```

**EXAMPLE 2**

**Separation and purification of rSIFN-co**

1. Fermentation

Inoculate the recombinant strain in LB media, shaking (200

rpm) under 37°C overnight (approximate 18 h), then add 30% glycerol to the fermentation broth to get final concentration of 15%, allotted to 1 ml tube and kept in -20°C as seed for production.

Add 1% of the seed to LB media, shaking (200 rpm) under 37°C overnight to enlarge the scale of the seed, then add to RM media with a ratio of 10%, culturing under 37°C. Add arabinose (20% solution) to 0.02% as an inductor when the OD600 reaches about 2.0. 4 hours after that, stop the culture process, collect the bacteria by centrifuge, resuspend the pellet with buffer A, and keep in -20°C overnight. Thaw and break the bacteria by homogenizer, then centrifuge. Wash the pellet with buffer B, buffer C, and distilled water to get a relatively pure inclusion body.

2. Denaturation and renaturation

Dissolve the inclusion body in Guanidine-HCl (or urea) of 6 mol/L. The solution will be a little cloudy. Centrifuge it at a speed of 10000 rpm. Determine the protein concentration of the supernatant. This supernatant is called "denaturation solution." Add the denaturation solution to renaturation buffer, and keep the final protein concentration under 0.3 mg/ml. It is better to add the totally denaturation solution in three steps instead of one step. Keep the solution overnight under 4°C. Afterwards, dialyze 10 mol/L,5 mol/L PB buffer and distilled water, then adjust its pH by 2 mol/L HAc-NaAc. Let it stand, then filtrate.

3. Purification

POROS HS/M anion exchange chromatography:

Equivalent column with 20 mmol/L HAc-NaAc(pH 5.0)

↓

Load samples at a speed of 30 ml/min

↓

Wash with 20 CV 20 mmol/L HAc-NaAc(pH 5.0)

↓

5 CV of 0.15 mol/L NaCl+20 mmol/L HAc-NaAc(pH 5.0)wash

↓

3 CV of 0.18 mol/L NaCl+20 mmol/L HAc-NaAc(pH 5.0)wash

↓

0.25 mol/L NaCl + 20 mmol/L HAc-NaAc(pH 5.0)elute target protein

Chelating sepharose™ fast flow: Add PB buffer of 0.2 mol/L(pH 6.6)and NaCl of 4 mol/L in the solution from HS to adjust solution pH to pH 6.0 and NaCl concentration to 1 mol/L.

Column with buffer D

↓

Loading at a rate of 1 ml/min

↓

Wash with buffer E

↓

Wash with buffer F

↓

Elute with buffer G

Condense the eluted solution by POROS HS/M. Sometimes a purification by sephacryl S-100 step can be added to meet stricter purity requirements.

Note:

Buffer A: 100 mmol/L Tris-HCl,pH 7.5—10 mmol/L EDTA—100 mmol/L NaCl

Buffer B: 50 mmol/L Tris-HCl,pH 7.5—1 mol/L Urea—10 mmol/L EDTA—0.5% Triton X-100

Buffer C: 50 mmol/L Tris-HCl,pH 7.5—2 mol/L Urea—10 mmol/L EDTA—0.5% Triton X-100

Buffer D: 1 mol/L NaCl ---50 mmol/L $Na_2HPO_4$ (pH 5.5)

Buffer E: 1 mol/L NaCl ---50 mmol/L $Na_2HPO_4$ (pH 5.0)

Buffer F: 1 mol/L NaCl ---50 mmol/L $Na_2HPO_4$ (pH 4.0)

Buffer G: 1 mol/L NaCl ---50 mmol/L $Na_2HPO_4$ (pH 3.6)

Renaturation buffer: 0.5 mol/L Arginine—150 mmol/L Tris-HCl, pH 7.5—0.2 mmol/L EDTA

**LB Media**: 1 L

| | |
|---|---|
| Tryptone | 10 g |
| Yeast extracts | 5 g |
| NaCl | 10 g |

**RM Media**: 1 L

| | |
|---|---|
| Casein | 20 g |
| MgCl | 1 mmol/L (0.203 g) |
| $Na_2HPO_4$ | 4 g; |
| $KH_2PO_4$ | 3 g, |
| NaCl | 0.5 g |
| $NH_4Cl$ | 1 g |

After purification, the buffer was changed to PBS (pH 7.0) along with the step of condensing by POROS HS/M. This is called the "Protein Stock Solution." It can directly used in the preparation of injections or sprays, or stored at 2-8°C.

Formula for injection:

| | Solution | Lyophilized powder |
|---|---|---|
| Solution of rSIFN-co | 34.5 μg/ml | 34.5 μg/ml |
| PB (pH7.0) | 25mmol/L | 10mmol/L |

| | | |
|---|---|---|
| Glycine | --- ---- -- | 0.4mol/L |
| NaCl | 0.1mol/L | ---- --- --- |

For spray:

| | |
|---|---|
| EDTA | 0.01% |
| Tween 80 | 0.05% |
| Trisodium citrate | 10mmol/L |
| Glycerol | 1.26% |
| Sodium Chloride | 0.03% |
| Phenylmethanol | 0.5% |
| HSA | 0.1% |
| rSIFN-co | 10 μg/ml |

**QUALITY CONTROL PROCESS**

During purification tests for protein content, protein purity, specific activity and pyrogen are conducted after each step. When the stock solution is obtained, all the tests listed in the table are done one after the other.

The quality of the product is controlled according to "Chinese Requirements for Biologics"

1. Original protein solution

Lowry

| Item of Test | Method |
|---|---|
| **Protein Stock Solution:** | |
| Test for Protein Content | Lowry |
| Test for Protein Purity | Non-reductive SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis )<br>HPLC Analysis |
| Test for Molecular Weights | Reductive SDS-PAGE |
| Test for Specific Activity | According to Method in "Specific Activity Test of Interferon |
| Test for Leftover Exogenetic DNA | Using DNA Labeling and Detection Kit |
| Test for Activity of Leftover Antibiotics | According to Method in "Chemical and Other Test Methods for Biologics" |
| Test for Bacterial Endotoxin | According to Method in "Requirements for Bacterial |

| | |
|---|---|
| | Endotoxin Test of Biologics" |
| Test for Isoelectronic Point | Isoelectric Focusing Electrophoresis |
| Test for Identify Characteristics of the Protein | UV spectrum (range of wavelength: 190-380nm) |
| | Peptide Mapping (hydrolyzed by pancreatic enzyme, analyzed by C-18 column) |
| | N-terminal Sequence Test |
| | C-terminal Sequence Test |
| | Circular Dichroism |
| | Amino Acid Analysis |
| | |
| **Semi-finished Product** | |
| Test for Bacterial Endotoxin | According to Method in "Requirements for Bacterial Endotoxin Test of Biologics" |
| | |
| **Product** | |
| Appearance Check | |
| Chemical | According to Method in "Chemical and Other Test Methods for Biologics" |
| Test for Specific Activity | According to Method in "Specific Activity Test of Interferon |
| Sterility Test | According to Method in "c" |
| Abnormal Toxicity Test | Test on Mouse |
| Pyrogen Test | According to Method in "Requirements for Pyrogen Test of Biologics" |
| Test for Stability of Product | |

Note: "Chemical and Other Test Methods for Biologics", "Requirements for Pyrogen Test of Biologics" and "Requirements for Bacterial Endotoxin Test of Biologics" all can be found in the "Chinese Requirements for Biologics." "Chinese Requirements for Biologics," PAN Zhengan, ZHANG Xinhui, DUAN Zhibing, et al. Chinese Biologics Standardization committee. Published by Chemical Industry Publishing Company, 2000.

**EXAMPLE 3**

**Stability of lyophilized Powd r of Recombinant Super-Compound Interferon Injection**

The stability experiments were carried out with samples of lyophilized powder of recombinant super-compound interferon (rSIFN-co) injection in two specifications and three batches. The experiments started on April, 2000.

**1. Sample Source**

Samples were supplied by Sichuan Huiyang Life-engineering Ltd., Sichuan Province. Lot: 990101-03, 990101-05, 990102-03, 990102-05, 990103-03, 990103-05

**2. Sample Specifications**

Every sample in this experiment should conform with the requirements in the table below.

Table 1 Standard of Samples in Experiment

| Items | Standards |
|---|---|
| 1. Appearance | white loose powder |
| 2. Dissolving time | dissolve rapidly in injection water( within 2 min) at room temperature |
| 3. Clarity | colorless liquid or with little milk-like glisten; should not be cloudy, impurity or with indiscernible deposit |
| 4. pH value | 6.5~7.5 |
| 5. Potency (IU/dose) | 80%~150% of indicated quantity ( 9μg:4.5 × $10^6$IU, 15μg: 7.5 × $10^6$IU) |
| 6. Moisture | no more than 3.0% ( W/W) |

**3. Experiment Content**

15.3.1 Test samples at 2~8℃: The test samples were put into a 2~8℃ refrigerator, then the above items of these samples were respectively tested in the $1^{st}$, $3^{rd}$, $6^{th}$, $9^{th}$, $12^{th}$, $18^{th}$, $24^{th}$, $30^{th}$, $36^{th}$ month. The results were recorded.

15.3.2 Test samples at 25°C: The test samples were put into a thermostat at 25°C, then the above items of these samples were respectively tested in the 1st, , 3rd , 6th, 9th, 12th, 18th, 24th, 30th month. The results were recorded.

15.3.3 Test samples at 37°C: The test samples were put into a thermostat at 37°C, then the above items of these samples were respectively tested in the 1st, , 3rd , 6th, 9th, 12th, 18th, 24th month. The results were recorded.

**4. Results and Conclusion**

1) At 37°C, according to data collected at designated points during testing and compared with data before testing, the potency began descending from the 6th month and the changes in the three batches were similar. The appearance of other items had no changes.

2) At 25°C, according to data collected at designated points during testing and compared with data before the testing, the potency only had a little change, and the changes in the three batches were similar. The appearance of other items had no changes.

3). At 2-8°C, according to data collected at designated points during testing and compared with data before testing, the potency of the three batches all were stable. The appearance of other items also had no changes.

In conclusion, it is suggested that the lyophilized powder of recombinant super-compound interferon for injection should be better stored and transported at low temperatures. Without such conditions, the product can also be stored for short periods (i.e. 3 months) at room temperature.

**EXAMPLE 4**

**rSIFN-co inhibits HBV-DNA duplication and secretion of HBsAg and HBeAg.**

*Materials*

Solvent and Dispensing Method: Add 1ml saline into each vial, dissolve, and mix with MEM culture medium at different concentrations. Mix on the spot.

Control drugs: IFN-α2b (Intron A) as lyophilized powder, purchased from Schering Plough. $3\times10^6$U each, mix to $3\times10^6$IU/ml with culture medium; Infergen® (liquid solution) , purchased from Amgen, 9μg, 0.3ml each, equal to $9X10^6$IU, and mix with $9X10^6$IU/ml culture medium preserve at 4°C; 2.2.15 cell: 2.2.15 cell line of hepatoma (Hep G2) cloned and transfected by HBV DNA, constructed by Mount Sinai Medical Center.

Reagent: MEM powder, Gibco American Ltd. cattle fetal blood serum, HycloneLab American Ltd. G-418(Geneticin); MEM dispensing, Gibco American Ltd.; L-Glutamyl, imported and packaged by JING KE Chemical Ltd.; HBsAg and HBeAg solid-phase radioimmunoassay box, Northward Reagent Institute of Chinese Isotope Ltd.; Biograncetina, Northern China Medicine; And Lipofectin, Gibco American Ltd.

Experimental goods and equipment: culture bottle, Denmark Tunclon™; 24-well and 96-well culture board, Corning American Ltd.; Carbon Dioxide hatching box, Shel-Lab American Ltd.; MEM culture medium 100ml: 10% cattle fetal blood serum, 3% Glutamyl1%, G418 380μg/ml, biograncetina50U/ml.

Method:

2.2.15 cell culture: Added 0.25% pancreatic enzyme into culture box with full of 2.2.15 cell, digest at 37°C for 3 minutes, and add culture medium to stop digest and disturb it to disperse the cells, reproduce with ratio of 1:3. They will reach full growth in 10 days.

Toxicity test: Set groups of different concentrations and a

control group in which cell is not acted on with medicine. Digest cell, and dispense to a 100,000 cell/ml solution. Inoculate to 96-well culture board, 200µl each well, culture at 37°C for 24h with 5% $CO_2$. Test when simple cell layer grows.

Dispense rSIFN-co to $1.8\times10^7$IU/ml solution than prepare a series of solutions diluted at two-fold gradients. Add into 96-well culture board, 3 wells per concentration. Change the solution every 4 days. Test cytopathic effect by microscope after 8 days. Fully destroy as 4, 75% as 3, 50% as 2, 25% as 1, zero as 0. Calculate average cell lesion and inhibition rate of different concentrations. Calculate TC50 and TC0 according to the Reed Muench method.

$$TC50 = \text{Antilog}\ (B + \frac{50-B}{A-B} \times C)$$

A=log >50 % medicine concentration, B=log<50 %medicine concentration, C=log dilution power

Inhibition test for HBeAg and HBsAg: Separate into positive and negative HBeAg and HBsAg contrast groups, cell contrast group and medicine concentration groups. Inoculate 700,000 cells/ml of 2.2.15 cell into 6-well culture board, 3 ml each well, culture at 37°C for 24h with 5% $CO_2$, then prepare 5 gradiently diluted solutions with 3-fold as the grade (Prepare 5 solutions, each with a different protein concentration. The concentration of Solution 2 is 3 times lower than that of Solution 1, the concentration of Solution 3 is 3 times lower than that of Solution 2, etc.) $4.5\times10^6$IU/ml, $1.5\times10^6$IU/ml, $0.5\times10^6$IU/ml, $0.17\times10^6$1U/ml, and $0.056\times10^6$1U/ml, 1 well per concentration, culture at 37°C for 24h with 5% $CO_2$. Change solutions every 4 days using the same solution. Collect all culture medium on the $8^{th}$ day. Preserve at -20℃ Repeat test 3 times to estimate HBsAg and HBeAg with solid-phase radioimmunoassay box (Northward Reagent Institute of Chinese Isotope Ltd.). Estimate cpm

value of each well with a γ- accounting machine.

Effects calculation: Calculate cpm mean value of contrast groups and different-concentration groups and their standard deviation, P/N value such as inhibition rate, IC50 and SI.

1) Antigen inhibition rate (%) = $\frac{A-B}{A} \times 100$

A = cpm of control group; B = cpm of test group;

2) Counting the half-efficiency concentration of the medicine

$$\text{Antigen inhibition IC50} = \text{Antilog}\ (B + \frac{50-B}{A-B} \times C)$$

A=log>50% medicine concentration, B=log<50 % medicine concentration, C=log dilution power

3) SI of interspace-conformation changed rSIFN-co effect on HBsAg and HBeAg in 2.2.15 cell culture:

$$SI = \frac{TC50}{IC50}$$

4) Estimate the differences in cpm of each dilution degree from the control group using student *t* test

Southern blot: (1) HBV-DNA extract in 2.2.15 cell: Culture cell 8 days. Exsuction culture medium (Separate cells from culture medium by means of draining the culture medium.). Add lysis buffer to break cells, then extract 2 times with a mixture of phenol, chloroform and isoamyl alcohol (1:1:1), 10,000g centrifuge. Collect the supernatant adding anhydrous alcohol to deposit nucleic acid. Vacuum draw, re-dissolve into 20μlTE buffer. (2) Electrophoresis: Add 6XDNA loading buffer, electrophoresis on 1.5% agarose gel, IV/cm, at fixed pressure for 14-18h. (3) Denaturation and hybridization: respectively dip gel into HCl, denaturaion buffer and neutralization buffer. (4) Transmembrane: Make an orderly transfer of DNA to Hybond-N membrane. Bake,

hybridize and expose with dot blot hybridization. Scan and analyze relative density with gel-pro software. Calculate inhibition rate and IC50.

**Results**

Results from Tables 1, 2 and 3 show: After maximum innocuous concentration exponent culturing for 8 days with 2.2.15 cell, the maxima is 9.0 ± $0\times10^{6}$IU/ml average inhibition rate of maximum innocuous concentration rSIFN-co to HBeAg is 46.0±5.25% (P<O . 001), IC50 is $4.54\pm1.32\mathrm{X}10^{6}$IU/ml, SI is 3.96; rate to HBsAg is 44.8± 6.6%, IC50 is $6.49\pm0.42\times10^{6}$IU/ml, SI is 2.77. This shows that rSIFN-co can significantly inhibit the activity of HBeAg and HBsAg, but that the IFN of the contrast group and Infergen® cannot. It has also been proved in clinic that rSIFN-co can decrease HBeAg and HBsAg or return them to normal levels.

Table 1: Results of inhibition rate of rSIFN-co to HBsAg and HBeAg

First batch: (rSIFN-co)

| Inhibition effect to HBeAg | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Concentration ($\times 10^4$IU/ml) | First well | Second well | Third well | Inhibition rate | | | Average inhibition rate | Accumulation | 1-Accumulation | Accumulated inhibition rate |
| | | | | First well | Second well | Third well | | | | |
| 900 | 9026 | 8976 | 10476 | 0.436227 | 0.43935 | 0.345659 | 0.407079 | 0.945909 | 0.592921 | 0.614693546 |
| 300 | 9616 | 12082 | 10098 | 0.3993754 | 0.245347 | 0.369269 | 0.337997 | 0.5388299 | 1.254924 | 0.300392321 |
| 100 | 9822 | 16002 | 12800 | 0.386508 | 0.0005 | 0.2005 | 0.195836 | 0.200833 | 2.059088 | 0.08867188 |
| 33.33333 | 15770 | 19306 | 16824 | 0.014991 | 0 | 0 | 0.004997 | 0.0049969 | 3.054091 | 0.001633453 |
| 11.11111 | 19172 | 22270 | 18934 | 0 | 0 | 0 | 0 | 0 | 4.054091 | 0 |
| Control | Cell | 16010 | | Blank | 0 | | Dilution | 3 | IC50 | 602.74446016 |

| Inhibition effect to HBsAg | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Concentration ($\times 10^4$IU/ml) | First well | Second well | Third well | Inhibition rate | | | Average inhibition rate | Accumulation | 1-Accumulation | Accumulated inhibition rate |
| | | | | First well | Second well | Third well | | | | |
| 900 | 7706 | 7240 | 7114 | 0.342155 | 0.381936 | 0.392693 | 0.372261 | 0.922258 | 0.627739 | 0.595006426 |
| 300 | 8856 | 7778 | 9476 | 0.2439816 | 0.336008 | 0.191053 | 0.257014 | 0.5499972 | 1.370724 | 0.286349225 |
| 100 | 10818 | 10720 | 10330 | 0.07649 | 0.084856 | 0.118149 | 0.093165 | 0.292983 | 2.27756 | 0.113977019 |
| 33.33333 | 10744 | 11114 | 10570 | 0.082807 | 0.051221 | 0.097661 | 0.07723 | 0.1998179 | 3.20033 | 0.058767408 |
| 11.11111 | 10672 | 9352 | 10810 | 0.088953 | 0.201639 | 0.077173 | 0.122588 | 0.122588 | 4.077742 | 0.02918541 |
| Control | Cell | 11714 | | Blank | 0 | | Dilution | 3 | IC50 | 641.7736749 |

Second batch: (rSIFN-co)

| Inhibition effect to HBeAg | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Concentration ($\times 10^4$IU/ml) | First well | Second well | Third well | Inhibition rate | | | Average inhibition rate | Accumulation | 1-Accumulation | Accumulated inhibition rate |
| | | | | First well | Second well | Third well | | | | |
| 900 | 7818 | 8516 | 9350 | 0.554378 | 0.514592 | 0.467054 | 0.512008 | 1.371181 | 0.487992 | 0.737521972 |
| 300 | 10344 | 10628 | 9160 | 0.4103967 | 0.394209 | 0.477884 | 0.427497 | 0.8591731 | 1.060496 | 0.447563245 |
| 100 | 12296 | 14228 | 13262 | 0.299134 | 0.18901 | 0.244072 | 0.244072 | 0.4316522 | 1.816423 | 0.19201839 |
| 33.33333 | 15364 | 17414 | 16188 | 0.124259 | 0.00741 | 0.77291 | 0.069653 | 0.1876045 | 2.74677 | 0.063933386 |
| 11.11111 | 17386 | 13632 | 15406 | 0.009006 | 0.222982 | 0.121865 | 0.117951 | 0.117951 | 3.628819 | 0.03148073 |
| Control | Cell | 16962 | | Blank | 0 | | Dilution | 3 | IC50 | 365.9357846 |

| Inhibition effect to HBsAg | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Concentration ($\times 10^4$IU/ml) | First well | Second well | Third well | Inhibition rate | | | Average inhibition rate | Accumulation | 1-Accumulation | Accumulated inhibition rate |
| | | | | First well | Second well | Third well | | | | |
| 900 | 5784 | 6198 | 5792 | 0.498265 | 0.462353 | 0.497571 | 0.486063 | 0.893477 | 0.513937 | 0.634835847 |
| 300 | 7150 | 8534 | 8318 | 0.379771 | 0.259715 | 0.278452 | 0.30598 | 0.4074138 | 1.207957 | 0.252210647 |
| 100 | 9830 | 11212 | 10210 | 0.147294 | 0.027412 | 0.11433 | 0.096345 | 0.101434 | 2.111612 | 0.04583464 |
| 33.33333 | 13942 | 12368 | 13478 | 0 | 0 | 0 | 0 | 0.0050891 | 3.111612 | 0.001632835 |
| 11.11111 | 12418 | 11634 | 11352 | 0 | 0 | 0.015267 | 0.005089 | 0.005089 | 4.106523 | 0.001237728 |
| Control | Cell | | | Blank | 0 | | Dilution | 3 | IC50 | 611.0919568 |

Third batch: (rSIFN-co)

| Inhibition effect to HBeAg | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Concentration ($\times 10^4$IU/ml) | First well | Second well | Third well | Inhibition rate | | | Average inhibition rate | Accumulation | 1-Accumulation | Accumulated inhibition rate |
| | | | | First well | Second well | Third well | | | | |
| 900 | 9702 | 9614 | 8110 | 0.428016 | 0.433204 | 0.521872 | 0.461031 | 1.316983 | 0.538969 | 0.709599543 |
| 300 | 8914 | 10032 | 8870 | 0.4744723 | 0.40856 | 0.477066 | 0.453366 | 0.8559525 | 1.085603 | 0.440859127 |
| 100 | 16312 | 12688 | 13934 | 0.038321 | 0.251975 | 0.178517 | 0.156271 | 0.402586 | 1.929332 | 0.172641621 |
| 33.33333 | 15080 | 12814 | 13288 | 0.110954 | 0.244547 | 0.216602 | 0.190701 | 0.2463153 | 2.738631 | 0.082519158 |
| 11.11111 | 21928 | 15366 | 15728 | 0 | 0.094093 | 0.072751 | 0.0055615 | 0.055615 | 3.683017 | 0.014875633 |
| Control | Cell | 17544 | | Blank | 0 | | Dilution | 3 | IC50 | 382.0496935 |
| Inhibition effect to HBsAg | | | | | | | | | | |
| Concentration ($\times 10^4$IU/ml) | First well | Second well | Third well | Inhibition rate | | | Average inhibition rate | Accumulation | 1-Accumulation | Accumulated inhibition rate |
| | | | | First well | Second well | Third well | | | | |
| 900 | 5616 | 6228 | 5346 | 0.496864 | 0.442035 | 0.521054 | 0.486651 | 0.763125 | 0.513349 | 0.597838293 |
| 300 | 8542 | 8590 | 7096 | 0.234725 | 0.230425 | 0.364272 | 0.276474 | 0.2764738 | 1.236875 | 0.182690031 |
| 100 | 11420 | 11360 | 11394 | 0 | 0 | 0 | 0 | 0 | 2.236875 | 0 |
| 33.33333 | 12656 | 11582 | 13110 | 0 | 0 | 0 | 0 | 0 | | 0 |
| 11.11111 | 13142 | 12336 | 13342 | 0 | 0 | 0 | 0 | 0 | 4.236875 | 0 |
| Control | Cell | 11528 | | Blank | 0 | | Dilution | 3 | IC50 | 694.7027149 |

HBeAg: Average IC50: 450.2434 SD: 132.315479

HBsAg: Average IC50: 649.1894 SD: 42.29580

Table 2: Results of inhibition rate of Intron A(IFN-α2b) to HBsAg and HBeAg

| Inhibition effect to HBeAg | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Concentration ($\times 10^4$IU/ml) | First well | Second well | Third well | Inhibition rate | | | Average inhibition rate | Accumulation | 1-Accumulation | Accumulated inhibition rate |
| | | | | First well | Second well | Third well | | | | |
| 300 | 14918 | 11724 | 9950 | 0 | 0.029711 | 0.176529 | 0.068747 | 0.068747 | 0.931253 | 0.068746724 |
| 100 | 14868 | 16890 | 15182 | 0 | 0 | 0 | 0 | 0 | 1.931253 | 0 |
| 33.33333 | 16760 | 21716 | 16400 | 0 | 0 | 0 | 0 | 0 | 2.931253 | 0 |
| 11.11111 | 20854 | 15042 | 16168 | 0 | 0 | 0 | 0 | 0 | 3.931253 | 0 |
| 3.703704 | 12083 | 12083 | 12083 | 0 | 0 | 0 | 0 | 0 | 4.931253 | 0 |
| Control | Cell | 17544 | | Blank | 0 | | Dilution | 3 | IC50 | FALSE |
| Inhibition effect to HBsAg | | | | | | | | | | |
| Concentration ($\times 10^4$IU/ml) | First well | Second well | Third well | Inhibition rate | | | Average inhibition rate | Accumulation | 1-Accumulation | Accumulated inhibition rate |
| | | | | First well | Second well | Third well | | | | |
| 300 | 9226 | 8196 | 9658 | 0.152489 | 0.247106 | 0.521054 | 0.1708 | 0.189295 | 0.8292 | 0.185857736 |
| 100 | 10946 | 10340 | 10828 | 0 | 0.050156 | 0.364272 | 0.018495 | 0.0184947 | 1.810705 | 0.010110817 |
| 33.33333 | 12250 | 12980 | 13934 | 0 | 0 | 0 | 0 | 0 | 2.810705 | 0 |
| 11.11111 | 12634 | 12342 | 12000 | 0 | 0 | 0 | 0 | 0 | 3.810705 | 0 |
| 3.703704 | 10886 | 10886 | 10886 | 0 | 0 | 0 | 0 | 0 | 4.810705 | 0 |
| Control | Cell | 10886 | | Blank | 0 | | Dilution | 3 | IC50 | FALSE |

Table 3: Results of inhibition rate of Infergen® to HBsAg and HBeAg

First batch: (Infergen®)

| Inhibition effect to HBeAg | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Concentration ($\times 10^4$IU/ml) | First well | Second well | Third well | Inhibition rate | | | Average inhibition rate | Accumulation | 1-Accumulation | Accumulated inhibition rate |
| | | | | First well | Second well | Third well | | | | |
| 900 | 14172 | 12156 | 17306 | 0.091655 | 0.220869 | 0 | 0.104175 | 0.306157 | 0.895825 | 0.254710274 |
| 300 | 13390 | 12288 | 16252 | 0.1417767 | 0.212409 | 0 | 0.118062 | 0.2019827 | 1.777764 | 0.102024519 |
| 100 | 14364 | 18834 | 14194 | 0.079349 | 0 | 0.090245 | 0.056531 | 0.083921 | 2.721232 | 0.029916678 |
| 33.33333 | 15722 | 16034 | 16340 | 0 | 0 | 0 | 0 | 0.0273897 | 3.721232 | 0.007306592 |
| 11.11111 | 17504 | 17652 | 14320 | 0 | 0 | 0.082169 | 0.02739 | 0.02739 | 4.693843 | 0.005801377 |
| Control | Cell | 15602 | | Blank | 0 | | Dilution | 3 | IC50 | FALSE |

| Inhibition effect to HBsAg | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Concentration ($\times 10^4$IU/ml) | First well | Second well | Third well | Inhibition rate | | | Average inhibition rate | Accumulation | 1-Accumulation | Accumulated inhibition rate |
| | | | | First well | Second well | Third well | | | | |
| 900 | 12080 | 11692 | 12234 | 0 | 0.01275 | 0 | 0.00425 | 0.025163 | 0.99575 | 0.024647111 |
| 300 | 12840 | 11484 | 12350 | 0 | 0.030313 | 0 | 0.010104 | 0.0209125 | 1.985646 | 0.010422073 |
| 100 | 12894 | 14696 | 15086 | 0 | 0 | 0 | 0 | 0.010808 | 2.985646 | 0.003606955 |
| 33.33333 | 15032 | 12928 | 13020 | 0 | 0 | 0 | 0 | 0.0108081 | 3.985646 | 0.002704416 |
| 11.11111 | 11794 | 11984 | 11508 | 0.004137 | 0 | 0.028287 | 0.010808 | 0.010808 | 4.974837 | 0.002167838 |
| Control | Cell | 11843 | | Blank | 0 | | Dilution | 3 | IC50 | FALSE |

Second batch: (Infergen®)

| Inhibition effect to HBeAg | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Concentration ($\times 10^4$IU/ml) | First well | Second well | Third well | Inhibition rate | | | Average inhibition rate | Accumulation | 1-Accumulation | Accumulated inhibition rate |
| | | | | First well | Second well | Third well | | | | |
| 900 | 6278 | 6376 | 6408 | 0.200051 | 0.187564 | 0.183486 | 0.190367 | 0.274635 | 0.809633 | 0.253290505 |
| 300 | 7692 | 9092 | 6394 | 0.0198777 | 0 | 0.18527 | 0.068383 | 0.0842678 | 1.74125 | 0.046161005 |
| 100 | 8960 | 7474 | 8190 | 0 | 0.047655 | 0 | 0.015885 | 0.015885 | 2.725365 | 0.005794856 |
| 33.33333 | 8530 | 8144 | 9682 | 0 | 0 | 0 | 0 | 0 | 3.725365 | 0 |
| 11.11111 | 7848 | 7848 | 7848 | 0 | 0 | 0 | 0 | 0 | 4.725365 | 0 |
| Control | Cell | 7848 | | Blank | 0 | | Dilution | 3 | IC50 | FALSE |

| Inhibition effect to HBsAg | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Concentration ($\times 10^4$IU/ml) | First well | Second well | Third well | Inhibition rate | | | Average inhibition rate | Accumulation | 1-Accumulation | Accumulated inhibition rate |
| | | | | First well | Second well | Third well | | | | |
| 900 | 12364 | 12268 | 12274 | 0.036171 | 0.043655 | 0.043187 | 0.041004 | 0.140162 | 0.958996 | 0.12751773 |
| 300 | 11590 | 12708 | 13716 | 0.0965076 | 0.009355 | 0 | 0.035287 | 0.0991581 | 1.923709 | 0.0490186 |
| 100 | 12448 | 13468 | 13982 | 0.029623 | 0 | 0 | 0.009874 | 0.063871 | 2.913834 | 0.02144964 |
| 33.33333 | 12616 | 11346 | 12444 | 0.016526 | 0.115529 | 0.029935 | 0.053996 | 0.0539965 | 3.859838 | 0.013796309 |
| 11.11111 | 12828 | 12828 | 12828 | 0 | 0 | 0 | 0 | 0 | 4.859838 | 0 |
| Control | Cell | 12828 | | Blank | 0 | | Dilution | 3 | IC50 | FALSE |

Third batch: (Infergen®)

| Inhibition effect to HBeAg | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Concentration ($\times 10^4$IU/ml) | First well | Second well | Third well | Inhibition rate | | | Average inhibition rate | Accumulation | 1-Accumulation | Accumulated inhibition rate |
| | | | | First well | Second well | Third well | | | | |
| 900 | 7240 | 6642 | 6158 | 0.064599 | 0.14186 | 0.204393 | 0.136951 | 0.217399 | 0.863049 | 0.201211735 |
| 300 | 11072 | 8786 | 6902 | 0 | 0 | 0.108269 | 0.03609 | 0.0804479 | 1.82696 | 0.042176564 |
| 100 | 7016 | 9726 | 7552 | 0.09354 | 0 | 0.024289 | 0.039276 | 0.044358 | 2.787683 | 0.015663017 |
| 33.33333 | 7622 | 8866 | 8676 | 0.015245 | 0 | 0 | 0.005082 | 0.0050818 | 3.782601 | 0.001341671 |
| 11.11111 | 7740 | 7740 | 7740 | 0 | 0 | 0 | 0 | 0 | 4.782601 | 0 |
| Control | Cell | 7740 | | Blank | 0 | | Dilution | 3 | IC50 | FALSE |

| Inhibition effect to HBsAg | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Concentration ($\times 10^4$IU/ml) | First well | Second well | Third well | Inhibition rate | | | Average inhibition rate | Accumulation | 1-Accumulation | Accumulated inhibition rate |
| | | | | First well | Second well | Third well | | | | |
| 900 | 11048 | 11856 | 11902 | 0.04775 | 0 | 0 | 0.015917 | 0.015917 | 0.984083 | 0.015916796 |
| 300 | 13454 | 12896 | 11798 | 0 | 0 | 0 | 0 | 0 | 1.984083 | 0 |
| 100 | 12846 | 13160 | 12546 | 0 | 0 | 0 | 0 | 0 | 2.984083 | 0 |
| 33.33333 | 12680 | 12458 | 12360 | 0 | 0 | 0 | 0 | 0 | 3.984083 | 0 |
| 11.11111 | 11602 | 11602 | 11602 | 0 | 0 | 0 | 0 | 0 | 4.984083 | 0 |
| Control | Cell | 11602 | | Blank | 0 | | Dilution | 3 | IC50 | FALSE |

HBeAg: Average IC50: 0 SD: 0

HBsAg: Average IC50: 0 SD: 0

**EXAMPLE 5**

**Preparation of rSIFN-co**

Preparation of lyophilized injection

| | Lyophilized powder |
|---|---|
| Stock Solution of rSIFN-co | 34.5 μg/ml |
| PB (pH7.0) | 10mmol/L |
| Glycine | 0.4mol/L |

Preparation technique: Weigh materials according to recipe. Dissolve with sterile and pyrogen-free water. Filter through 0.22μm membrane to de-bacterialize, preserve at 6-10°C. Fill in vials after affirming it is sterile and pyrogen-free, 0.3 ml /vial or 0.5 ml/vial, and lyophilize in freeze dryer.

Preparation of liquid injection

| | Solution |
|---|---|
| Stock Solution of rSIFN-co | 34.5 μg/ml |
| PB (pH7.0) | 25mmol/L |
| NaCl | 0.1mol/L |

Preparation: Weigh materials according to recipe. Add to desired level with sterile and pyrogen-free water. Filter through 0.22μm membrane to de-bacterialize, preserve at 6-10°C. Fill in airtight vial after affirming it is sterile and non-pyrogen at 0.3 ml /vial or 0.5 ml/vial. Storage at 2-10°C, and protect from light.

**EXAMPLE 6**

**Acute Toxicity of rSIFN-co**

Treat mice with large dose (150μg/kg, equal to 1000 times of the normal dose per kilo used in treatment of adult patients) of rSIFN-co at one time by intramuscular injection. Then, observe and record their deaths and toxic reactions. Results show that: 24 hours after injection, no

abnormal reaction had been recorded. The organs of the animals which had been selected to be killed also had no signs of abnormal changes. Those remaining mice were all kept alive and were normal after two weeks. The weights of mice in the experimental group and control group all increased, and the ratio of increase had no obvious difference between the two groups (P>0.05) according to their weights on the fourteenth day. No abnormal changes were seen from the main organs of those mice after two weeks.

1. Experimental material

1.1 Animals

40 healthy adult mice, weighing 18-22g, half male and half female, qualified by Sichuan experiment animal control center.

2.2 Medicines

rSIFN-co (Provided by Sichuan Huiyang Life-engineering Ltd.) sterilized solution, 0.15 mg/ml, Lot: 981201

rSIFN-co was administered *i.m.* in saline.

2. Method

Separate the 40 mice into two groups randomly, one for experimental medicine, another for control. Inject medicines or saline at the same ratio (0.1 ml/10 g) through muscle to each mouse according to which group they belong. (150 µg/kg of rSIFN-co for experimental group; and saline for control group). After injection, observe and record acute toxicity shown in mice. Kill half of the mice (male and female each half) to check whether there were any abnormal pathologic changes in their main organs, such as heart, spleen, liver, lung, kidney, adrenal gland, stomach, duodenum, etc. after 24 hours. Those remains were kept and observed until the fourteenth day. Weigh all mice, kill them, and then observe the appearance of the organs listed above to see if there are any abnormalities. Take pathological tissue and examine it, using the examination to assess the difference in weight increases in the two

groups.

3. Results

Results show that there was no acute toxicity seen after all mice were treated with i.m. rSIFN-co with 150 µg/kg at a time, equal to 1000 times the normal dose per kilo used in treatment of adult patients. In the 14 days after injection, all mice lived well. They ate, drank, exercised, and excreted normally and showed normal hair conditions. None of them died. The observation of the main organs of the randomly selected mice shows no abnormal changes 24 hours after injection. 14 days after injection, all remaining mice were killed. Autopsies also showed no changes. The weights of mice in the two groups all increased, but no obvious difference was shown when accessed with statistic method ($p > 0.05$). See Table 1:

Table 1 Influence to weights of mice after injection of rSIFN-co

| Group | Dose | Animal | Weights before injection (g) | Weights after injection (g) | Increased value of weights (g) |
|---|---|---|---|---|---|
| Control | 0 | 20 | 19.8 ± 1.7 | 30.8 ± 2.8 | 11.0 ± 2.9 |
| rSIFN-co | 150 | 20 | 19.4 ± 1.7 | 32.1 ± 3.3 | 12.7 ± 4.3 |

3. Conclusion

Under conditions of this experiment, there were no toxic reactions in all mice after injection of rSIFN-co with 150 µg/kg. The conclusion can be reached that the maximum tolerable dose of i.m. in mice is 150 µg/kg, which is equal to 1000 times the normal dose per kilo used in treatment of adult patients.

**EXAMPLE 7**

**The clinic effects of recombinant super-compound interferon (rSIFN-co)**

The recombinant super-compound interferon (rSIFN-co) is an

invention for viral disease therapy, especially for hepatitis. Meanwhile, it can inhibit the activity of EB viruses, VSV, Herpes simplex viruses, cornaviruses, measles viruses et al. Using Wish cells /VSV system as the assay for anti-virus activity, the results showed that: the other rIFN, was $0.9\times10^8$ IU/mg, Intron A was $2.0\times10^8$ IU/mg and rSIFN-co was $9\times10^8$ IU/mg. The anti-viral activity of rSIFN-co is much higher than those of the former two.

Under the permission of the State Food and Drug Administration (SFDA), People's Republic of China, the clinical trials have taken place in West China Hospital, Sichuan University, the Second Hospital of Chongqing Medical University, the First Hospital of School of Medical, Zhejiang University since the Feb 2003. The clinical treatment which focuses on the hepatitis B is conducted under the guidance of the mutilcenter, double-blind random test. IFN-α1b was used as control, and the primary results showed the following:

**The effect of rSIFN-co compared with IFN-α1b in the treatment of chronic active hepatitis B**

**1. Standard of patients selection:** The standard 1-4 are effective to both treatment with rSIFN-co (9μg) and IFN-α1b (5MU, 50μg), and the standard 1-5 are for rSIFN-co (15μg) treatment.

1). Age: 18-65

2). HBsAg test positive last over six months, HBeAg test positive, PCR assay, HBV-DNA copies $\geq10^5$/ml

3). ALT≥two times of the normal value

4). Never received IFN treatment; or those received the Lamividine treatment but failed or relapsed

5) Once received other IFNs (3MU or 5MU) treatment six months ago, following the standard of SFDA but failed or relapsed

**2. Evaluation of the effects:**

In reference to the recommendations from the Tenth China National Committee of Virus Hepatitis and Hepatopathy, the effects were divided into three degrees according to the ALT level, HBV-DNA and HBeAg tests.

Response: ALT normal level, HBV-DNA negative, HBeAg negative

Partial response: ALT normal level, HBV-DNA or HBeAg negative

Non response: ALT, HBV-DNA and HBeAg unchanged

The response and partial response groups consider as effective cases.

**3. Results of clinic trial:**

Group A: treatment with rSIFN-co(9μg)

Group B: treatment with IFN-α1b (5MU, 50 μg)

| Period | group | Medicine | cases | Effective Rate | HBsAg Transfer to negative rate | HBeAg Transfer to negative rate | HBV-DNA Transfer to negative rate | Heptal function Recover rate |
|---|---|---|---|---|---|---|---|---|
| 8-12 week | A | rSIFN-co(9μg) | 32 | 46.88 (15) | 9.38 (3) | 28.12 (9) | 37.50 (12) | 84.38 (27) |
| | B | IFN-α1b (5MU, 50 μg) | 32 | 21.88 (7) | 0.00 (0) | 9.38 (3) | 15.62 (5) | 56.25 (18) |
| 16-24 week | A | rSIFN-co(9μg) | 64 | 54.69 (35) | 7.81 (5) | 25.00 (16) | 34.38 (22) | 90.62 (58) |
| | B | IFN-α1b (5MU, 50 μg) | 64 | 25.00 (16) | 0.00 (0) | 9.38 (6) | 18.75 (12) | 78.13 (50) |

In Group C, the cases were chronic active hepatitis B treatment with other IFNs (3MU or 5MU) before but failed or relapsed and treated with rSIFN-co (15 μg), subcutaneous injection, every one day, last 24 weeks. The total cases are 13. After 12 weeks treatment, 7 of 13 (53.85%) were effective. 3 of 13 (23.08%) HBeAg transferred to negative; 7 of 13(53.85%) HBV-DNA transferred to negative; 11 of 13 (84.62%) hepal functions recovered to normal.

**4. The side effects of rSIFN-co compar d with IFN-α1b in th treatment**

The side effects of IFN include fever, nausea, myalgia, anorexia, hair lose, leucopenia and thrombocytopenia, etc. The maximum dose of IFN-α1b is 5MIU per time; the routine dose is 3 MIU. When taken the routine dose, 90% patients have I- II degree (WHO standard) side effects. They are fever lower than 38°C, nausea, myalgia, anorexia, etc. When taken at maximum dose, the rate of side effects do not rise obviously, but are more serious. The maximum dose of rSIFN-co is 24μg, subcutaneous injection, every one day for 3 months. The routine dose is 9μg. When routine doses were used, less than 50% patients have I-II degree (WHO standard) side effects, including fever below 38°C, nausea, myalgia, anorexia, leucopenia and thrombocytopenia slightly. With maximum dosage, about 50% patients suffered from leucopenia and thrombocytopenia after using **rSIFN-co** one month, but those side effects would disappear after stopping treatment for one week. It is safe for continue use.

**The observations of rSIFN-co treat hepatitis C**

**1. Standard of patient's selection**

1) age: 18-65

2) HCV antibody positive

3) ALT≥1.5 times of the normal value, last more than 6 months

**2. Evaluation of the effects:**

Referring to the standard of Infergen® for treatment of hepatitis C and according to the ALT level and HCV-RNA test, divided the effects into three degree:

Response: ALT normal level, HCV-RNA negative

Partial response: ALT normal level, HCV-RNA unchanged

Non response: ALT and HCV-RNA unchanged

**3. Effects in clinic**

The clinical trial was done at the same time with hepatitis B treatment. 46 cases received the treatment, 9 μg each time, subcutaneous injection, every day for 24 weeks. After treatment, 26 of 46 (56.52%) have obvious effects, 12 of 46

(26.08%) HCV-RNA transferred to negative, 26 of 46 (56.52%) hepal functions recovered to normal.